IN VIVO MODEL FOR EXPERIMENTAL MANIPULATION OF CALCIFIED TISSUES AND ASSOCIATED SOFT TISSUES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to an *in vivo* model for experimental manipulation of calcified tissues and associated soft tissues, usage thereof in drug screening for the treatment of various diseases, such as osteoporosis, and neoplastic transformation; usage thereof for study of toxicity of biomaterials; usage thereof to study cell function, inflammation, immune response, extracellular matrix events such as mineralization and cell signaling apoptosis among others, and usage thereof to test the efficiency of gene transfer construct.

(b) Description of Prior Art

Tooth development is mediated by reciprocal inductive interactions between neural crest-derived ectomesenchyme cells and the oral epithelium (reviewed in Thesleff et al. 1996). Cells of epithelial origin differentiate into ameloblasts which synthesize and secrete the noncollagenous matrix of enamel, and ectomesenchymal cells give rise to odontoblasts which produce the collagen-containing dentin matrix. Epithelial and/or ectomesenchymal cells may also differentiate into cementoblasts which deposit cementum, a tissue which comprises both noncollagenous and collagenous proteins (reviewed in Bosshardt and Nanci 1998). Furthermore, the eruption and growth of the tooth involves the participation of surrounding/supporting bone and periodontal tissues. More specifically, alveolar bone remodeling has been shown to occur around the apical portion of the rat incisor allowing for posterior growth of the tooth and periodontal ligament restructuring along the root analogue surface for eruption of the tooth.

Formation and mineralization of all the above calcified tissues results from a tightly-regulated series of cellular events and interactions between the organic and inorganic phases of the extracellular matrix.

The continuously erupting rat incisor has been extensively used to study the cellular and extracellular matrix events involved in odontogenesis since all stages of development can be found in a single tooth and it exhibits numerous similarities to human tooth formation. Facing epithelial and mesenchymal cells undergo a series of differentiation and phenotypic changes which have been precisely mapped along the linear axis of the tooth (Smith and Nanci 1989). Anatomically, much of the rat incisor lies in the body of the mandible. In addition, its odontogenic organ, responsible for renewal of all epithelial cells of the tooth organ, is situated in a very active but secluded area, making the incisor an excellent model for local and selective targeting of dental tissue cells.

Despite the advantages offered by the rat incisor, there only have been limited attempts at developing experimental approaches for direct manipulation of the cellular and matrix events in this tooth and supporting tissues. Some experiments have involved removal of blocks of bone and apical portions of the incisor to study cell activity during bone remodeling and tooth eruption (Redondo et al. 1995; Berkovitz and Thomas 1969; Berkovitz 1971a, b). Others have implicated the surgical creation of a hole ('window') through the alveolar bone at the labial surface of the tooth in order to get direct access to the underlying tissues (McKee and Warshawsky 1984; Eisenmann et al. 1989; McKee 1993; McKee and Nanci 1996a). These approaches were designed to target only secretory and maturation stage ameloblasts as well as the adjacent enamel. However, due to the confine and narrow

space separating the alveolar bone and the underlying enamel organ, previous surgical procedures caused considerable damage to tooth structure and require waiting for undamaged tissues to move forward under the window before further experimentation can be accomplished. Moreover, they do not allow targeting of the precursor cells located at the apical end of the tooth, where inductive molecular events take place, and are thus restricted to specific developmental stages of the enamel organ. Most importantly, since enamel forms in a secluded environment regulated and maintained, at least in part by ameloblasts, any interruption in the integrity of the enamel organ will inevitably lead to an alteration of the physiological parameters of enamel.

Despite efforts to elucidate the regulatory role of matrix molecules and growth factors in tooth and bone formation, there are still many questions regarding the molecular mechanisms underlying their formation.

It would be highly desirable to be provided with an *in vivo* model for experimental manipulation of calcified tissues and associated soft tissues.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide with the use of a rodent's mandibular incisor as an experimental model for the local and selective targeting of the odontogenic organ and its associated periodontal tissues. A surgical technique was developed to create a 'window' in the alveolar bone overlying the apex of the rodent incisor to allow direct diffusion of specific experimental agents. While direct deposition in the window is possible in some circumstances, an osmotic minipump is preferred to deliver the specific experimental agents in the window.

Vinblastine sulfate and 2 tracer molecules, fetuin-gold and albumin tagged with dinitrophenol, were

utilized to validate the efficiency of the surgical approach in targeting cells of the tooth organ. Minipumps have previously been used in dogs to deliver bafilomycin A_1, an inhibitor of vacuolar H+ - ATPases in osteoclasts, in order to block alveolar bone resorption and tooth eruption (Sundquist and Marks 1994; Marks and Sundquist 1995). They are advantageous since they can deliver, in a controlled and continuous manner, relatively large amounts of experimental agents, as compared to microinjection. This *in vivo* experimental model may prove advantageous for future experiments in which various drugs and molecular probes are applied to elucidate the cellular and biochemical events regulating calcified tissue formation and their alteration in disease.

In accordance with the present invention, there is provided an *in vivo* rodent model for testing of a compound in locally targeted odontogenic organ and associated periodontal tissues, comprising a mandibular incisor and a window in an alveolar bone overlying the incisor's apex or along radicular surface of the incisor, wherein said window is adapted for direct diffusion of a tested compound; and wherein a reduction in parameters consisting in histological, histomorphometric, eruption rate and matrix organization of said incisor compared to a normal incisor is indicative of a compound interfering with cellular or biochemical events involved in tissue formation of said incisor.

In accordance with another embodiment of the present invention, the window is further adapted to receive an osmotic pump for delivery of said tested compound.

In accordance with the present invention, there is provided a drug screening for a potential bone disease therapy compound, which comprises the steps of:

a) administering said compound to the model of the

present invention;

b) determiming histological and histomorphometric parameters of alveolar bone of the model's incisor and comparing to a normal incisor; wherein increase in anabolic activities or decrease in catabolic activities are indicative of a potential bone disease therapy compound.

The bone diseases include, without limitation, osteoporosis.

In accordance with the present invention, there is provided a drug screening for a compound inducing tissue repair, which comprises the steps of:

a) administering said compound to the model of the present invention;

b) determiming histological and histomorphometric parameters of alveolar bone and other tisues of the model's incisor and comparing to a normal incisor; wherein increase in anabolic activities or decrease in catabolic activities are indicative of a compound inducing tissue repair.

The preferred compound is a growth factor or a combination of growth factors.

In accordance with the present invention, there is provided a drug screening for a potential anti-cancer therapy compound, which comprises the steps of:

a) administering said potential anti-cancer therapy compound to the model of the present invention,

b) determiming eruption rate of the model's incisor and comparing to normal eruption rate of an incisor; wherein a decrease in growth rate is indicative of a potential anti-cancer therapy compound in controlling cell division.

In accordance with the present invention, there is provided a test to determine the role of extracellular matrix molecules, which comprises the steps of:

a) administering to the model of the present invention using a minipump drugs that affect secretion and postranslational modifications of said extracellular matrix molecules and/or antibodies or antagonists thereof;

b) determiming histological and histomorphometric parameters of alveolar bone and other tissues of the model's incisor and comparing to a normal incisor; wherein cell organization and behavior, extracellular matrix organization, and degree of mineral deposition are indicative of a drug interfering with said extracellular matrix molecules.

In accordance with the present invention, there is provided a test for the immune response, which comprises the steps of:

a) administering selected pathogens to the model of the present invention using a minipump;

b) determiming growth profile of pathogen, tissue alterations, inflammatory cell infiltration are indicative of a pathogen inducing an immune response.

In accordance with the present invention, there is provided a test for toxicity of certain elements used for making biomaterials such as metals, which comprises the steps of:

a) administering concentrations of metal ions to the model of the present invention using a minipump;

b) determiming cell death, cell division and matrix production of the bone and tooth tissues of the model's incisor and comparing to a normal incisor; wherein increase in anabolic activities or decrease in catabolic activities are indicative of a toxic metal.

In accordance with the present invention, there is

provided a test for the efficiency of genetic material transfer, which comprises the steps of:

a) administering an expression construct which encodes for a marker gene and/or a gene of interest to the model of the present invention using a minipump;

b) determiming expression of said marker to assess efficiency of said expression construct to transduce the tooth organ and associated tissues.

In accordance with the present invention, there is provided a method of gene therapy, which comprises the steps of:

a) administering an expression construct which encodes for a gene of interest to the model of the present invention using a minipump;

b) inducing or repressing expression of a gene product of said gene.

In accordance with the present invention, there is provided a method of producing a non-human mammal knock-out model, whose germ cells and somatic cells are modified to express at least one gene or an allelic variant of said gene which comprises introducing an expression construct which encodes for a gene of interest or a deletion of said gene to the model of the present invention using a minipump into the mammal, or an ancestor of the mammal, at an embryonic stage.

For the purpose of the present invention the following terms are defined below.

The term "rodent" is intended to mean an animal having a continuously erupting tooth or a mandibular incisor, which includes, without limitation, rat, rabbit, mouse, hamster, guinea pig, squirrel, beaver, among others. The preferred rodent is the rat.

The term "expression construct" is intended to mean any vehicule, such as vectors (sense or anti-sense),

plasmids, lipids, or naked DNA. Vectors may include, without limitation, adenovirus vectors.

The term "gene product" is intended to mean any product encoded directly or indirectly from the gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A illustrates a photograph of the first incision (1) made through the skin and muscle for drilling a surgical window (arrow) through the alveolar bone overlying the connective tissue surrounding the apex of a rat incisor;

Fig. 1B illustrates a photograph of the second skin incision (2) for the dorsal positioning of a osmotic minipump;

Fig. 1C illustrates a microradiograph showing the position of the metal tip of the catheter with respect to the apical portion (arrowhead) of the incisor;

Fig. 1D illustrates a photograph of an hemimandible with the metal tip of the catheter fitted into the bony window (arrowhead);

Fig. 2 illustrates a scanning electron microscope image of the hole drilled through the mandibular bone;

Fig. 3 illustrates schematic illustrations of the posterior aspect of the hemimandible showing the relationship of the apical end of the incisor to the bony window (top) and the plane of section, passing along the longitudinal axis of the tooth and across the window (bottom), used to obtain Figs. 4 and 5;

Figs. 4A-E illustrate light micrographs illustrating the relation of the bony window to the enamel organ (EO) of the incisor;

Figs. 5A-F illustrate micrographs illustrating the alterations induced by infusion of vinblastine sulphate for 3 days;

Figs. 6A-B illustrate immunocytochemical

preparations with anti-amelogenin (AMEL) antibody;

Figs. 7A-B illustrate comparative immunocytochemical preparations with anti-amelogenin (AMEL) antibody showing the apical portion of secretory stage ameloblasts from (A) the vinblastine treated and (B) untreated contralateral incisor;

Figs. 8A-B illustrate immunocytochemical preparations illustrating the distribution of infused albumin-dinitrophenol (ALB-DNP) in the connective tissue surrounding the enamel organ;

Figs. 9A-B illustrate albumin-dinitrophenol (ALB-DNP) is immunodetected in osteoid of the (A) treated hemimandible but not on the (B) contralateral side, indicating that there has been minimal recirculation of the tracer during the 24 hour infusion interval;

Figs. 10A-B illustrate immunocytochemical preparations illustrating the presence of albumin-dinitrophenol (ALB-DNP) tracer in multivesicular bodies (mvb) of early secretory stage ameloblasts;

Figs. 11A-B illustrate some of the infused albumin-dinitrophenol (ALB-DNP) tracer diffuses through the apical foramen of the incisor into the pulp chamber where it is taken up into endosomal/lysosomal elements (arrowheads) of (A) odontoblasts and (B) resident mesenchymal cells;

Figs. 12A-B illustrate fetuin-gold complex (A) binds to the extracellular matrix (asterisks) and is heavily taken up into endosomal/lysosomal elements (arrowheads) by inflammatory cells such as (A) neutrophils and (B) macrophages at the site of the bony window;

Figs. 13A-B illustrate that despite their relatively large size, fetuin-gold complexes can be found (A) along the enamel organ and (B) into the pulp where they are endocytosed and directed to endosomal/lysosomal

elements (arrowheads) of connective tissue cells; and

Fig. 14 illustrates preliminary results of gene transfer using adenovirus gene transfer system.

DETAILED DESCRIPTION OF THE INVENTION

The tooth organ is extensively used in developmental biology to study events mediating organogenesis and cell differentiation. It also represents an advantageous system for the study of the various cellular and extracellular matrix events regulating the formation of both collagenous and non-collagenous calcified tissues. This article describes a surgical approach to access and experimentally manipulate *in vivo* the tooth organ and supporting tissues of the rat incisor. Using a dental drill, a 'window' was created through the alveolar bone on the buccal aspect of the hemimandible at the apical end of the incisor. It is at this site that epithelial and mesenchymal precursors are situated and undergo cellular differentiation to give rise to cells of the odontogenic organ. Active bone remodeling is also observed in this area to accommodate posterior growth of the tooth. An osmotic minipump connected to the bony window through an outlet catheter was used for the controlled and continuous administration of experimental agents over a predetermined period of time. In order to validate the model, vinblastine sulfate, fetuin-gold and dinitrophenylated albumin were thus infused. The animals were then sacrificed and the hemimandibles were processed for histological and immunocytochemical analyses. The effects of the drug and the presence of tracers were restricted to the treated hemimandible and were found in the enamel organ and pulp, as well as in the tooth supporting tissues. Cellular changes typically associated with the administration of vinblastine were obtained, and tracers were localized

both in the extracellular milieu and within the endosomal/lysosomal elements of cells. These results suggest that this new surgical approach could serve as an advantageous *in vivo* model in which various chemical agents, therapeutic drugs, as well as molecular probes are locally administered to study the molecular events regulating calcified tissue formation.

Surgical procedure

Male Wistar rats weighing 100 ± 10g were anaesthetized with an intraperitoneal injection of 0.06 ml sodium pentobarbital. The vestibular surface of the right mandibular ramus was surgically exposed as follows (Fig. 1). An incision about 8 mm in length was made through the skin with fine scissors to expose the muscle layer underneath, according to an imaginary line joining the auditory meatus and the lip commissure. The fibers of the masseter were separated along their longitudinal axis using a scalpel blade. A periosteal separator was then used to elevate the periosteum and expose the underlying bony surface of the ramus. The musculature was retracted with a plastic ring made from an embedding BEEM capsule size 1. The surgical area was kept moist with rinses of physiological saline. A slow-speed dental drill equipped with a carbide round bur size 6 was used to create a hole through the alveolar bone (Fig. 1A).

In accordance with another embodiment of the present invention, the hole may also be located at any point along the rooth analogue surface of the incisor to allow direct targetting to specific tissues and regions of the incisor without causing any damages to those tissues.

The bony window was placed approximately 2 mm anterior to the posterior border of the ramus and slightly superior to the bony elevation at the apical end

of the incisor. Penetration through the alveolar bone into the periodontal space around the apex was established by slight bleeding upon breakthrough. The bur was then removed and a cotton swab was placed over the hole until the bleeding stopped. A third incision through the skin in the neck area was made to accommodate an Alzet 1003D or 2001D osmotic minipump (Alza Corporation, Palo Alto, CA; Fig. 1B). The pump catheter passes below the masseter. The 1003D model has a capacity of 90 µl and a flow rate of 1 µl/hr for 3 days while the 2001D has a capacity of 234 µl and a flow rate of 8 µl/hr for 24 hours. The pump was tunneled into a subcutaneous pouch on the back of the animal and connected to the bony hole using a vinyl tubing and a metal catheter made from a 20G1 syringe needle. The tubing was passed underneath the masseter muscle and through the neck area. Histoacryl glue and bone cement (Zimmer, Warsaw, IN) were used to retain the metal catheter in place. The animals were then sutured and the surgical site was cleaned and disinfected with 70% ethanol. Following surgery, X-ray photographs of the rat mandibles were taken in order to verify that the catheter was well in place (Fig. 1C) and the rats were allowed to recover under observation. Some of the animals received 0.01ml of buprenorphine hydrochloride 0.3mg/ml as analgesic immediately following surgery. All animal procedures and experimental protocols described above were in strict accordance with guidelines of the Ethic Committee of Experimental Procedure on Animal of the University of Montreal.

Administration of vinblastine sulfate

Six rats were implanted with Alzet osmotic minipumps model 1003D (Alza) filled with a solution of 0.17 mg/ml of vinblastine sulfate in physiological saline. The minipumps were connected to the vinyl tubing, also filled with the drug, and incubated in sterile

saline at 37□C for 3 hours prior to placement as described above.

Administration of tracers

Groups of 2 rats were implanted with saline preincubated Alzet 2001D minipumps (Alza) filled with fetuin-gold or DNP-tagged albumin for 24-hour infusion. The fetuin-gold complex (particles of ~15 nm in diameter; 25 µg/ml) was purchased from E.Y. Laboratories, Inc., San Mateo, CA. Bovine serum albumin was tagged with dinitrophenol (DNP) using the method of Little and Eisen (1967).

Tissue processing

On the third day of infusion of vinblastine sulfate and at 24 hours for the tracers, the animals were anaesthetized with an intraperitoneal injection of 0.25 ml of 20% chloral hydrate and sacrificed by intravascular perfusion through the left ventricle. The vasculature was prerinsed with lactated Ringer's solution for about 30 seconds (until the liver blanched) followed by perfusion with a fixative solution consisting of 1% glutaraldehyde in 0.08 M sodium cacodylate buffer containing 0.05% $CaCl_2$ pH 7.3 for 20 minutes. Both hemimandibles were dissected out and immersed in the fixative overnight at 4°C. They were then washed in 0.1 M sodium cacodylate buffer containing 0.05% $CaCl_2$ pH 7.3 and decalcified in 4.13% EDTA for 14 days at 4°C (solution was changed every 2 days). The hemimandibles were subdivided into segments and washed again in 0.1M sodium cacodylate buffer. Half of the segments were postfixed with 2% potassium ferrocyanide-reduced osmium tetroxide for 2 hours at 4°C. All segments (osmicated and non osmicated) were then dehydrated in a graded alcohol series and embedded in LR White resin or dehydrated in a graded acetone series and embedded in Taab epoxy resin. Liver, duodenum, kidney,

and parotid gland were also harvested and processed for embedding in LR White or epoxy resin.

Each tooth segment was oriented for sectioning along its longitudinal axis (see schematic illustration in Fig. 3). One µm-thick sections were cut with glass knives on a Reichert-Jung Ultracut E ultramicrotome and stained with toluidine blue. Thin sections were cut with a diamond knife and mounted on 200 mesh nickel grids having a carbon-coated Formvar film. Selected sections were processed for postembedding colloidal gold immunocytochemistry for detection of amelogenin or DNP-tagged albumin while fetuin-gold was directly observed. All grids were stained with uranyl acetate and lead citrate for examination in a JEOL JEM-1200EX-II transmission electron microscope operated at 60 kV.

Immunolocalization of dinitrophenol-tagged albumin and amelogenin

Sections from osmicated samples were first treated with sodium metaperiodate for half an hour (LR White) or 1 hour (epoxy resin) and rinsed with distilled water. All sections were floated for 15 minutes onto a drop of 0.01 M phosphate-buffered saline (PBS) containing 1% ovalbumin (Oval). They were transferred onto a drop of rabbit anti-DNP antibody diluted 1:150 or recombinant M179 mouse amelogenin antibody diluted 1:300 for 1 hour, washed with PBS, and refloated on PBS-Oval for 15 minutes. Incubating the tissue sections with protein A-gold complex for 30 minutes then revealed antibody binding sites. For the detection of enamel proteins, a chicken egg yolk antibody to rat 24 kDa amelogenin was also used. Briefly, the sections were floated on a drop of a blocking solution [0.5% BSA, 0.1% Gelatin, 0.005% Tween 20 (Bio-Rad) and 0.5M NaCl dissolved in 0.01M PBS, pH 7.4] for 15 minutes. They were then incubated on a drop of a primary egg yolk antibody (diluted 1:150 in PBS 0.01M) for 3 hours, washed

with PBS and refloated on the blocking solution for another 15 minutes. Afterward, they were incubated with a rabbit anti-chicken IgG antibody (diluted 1:2000) for 1 hour. They were washed in PBS, refloated on the blocking solution for 15 minutes and incubated with protein A-gold complex for 30 minutes. For controls, grids were incubated with secondary antibody followed by protein A-gold, or protein A-gold alone. All incubation steps were carried out at room temperature. The grids were finally rinsed with PBS, distilled water, and then air dried.

Scanning electron microscopy

Two animals were sacrificed by anaesthetic overdose after creation of the bony window. The hemimandibles were dissected and cleaned of soft tissue, fixed overnight by immersion with 1% glutaraldehyde in 0.08 M cacodylate buffer containing 0.05% $CaCl_2$ pH 7.3 and washed in 0.1 M sodium cacodylate buffer. Residual soft tissue was removed by digestion with 6% sodium hypochlorite. They were then washed and kept in distilled water until observation in the humid state with an Hitachi S-3500N variable pressure scanning electron microscope operated in the backscattered mode at 20 kV and 40 Pa pressure.

Surgical procedure

Initial studies aimed at establishing the appropriate position of the bony window identified the posterior border of the ramus and the bony elevation overlying the apical end of the incisor as reliable reference points for drilling. Passing the minipump catheter below the masseter muscle and immobilization of its metal tip with Histoacryl glue and bone cement resulted in a firm anchorage. The positioning and stability of the catheters in the bony window were confirmed on X-rays (Fig. 1C). Visual inspection at

dissection time was also used to confirm that the catheter was still in position (Fig. 1D) and to rule out any blockage by tissue debris and blood clotting. These parameters allowed proper positioning of the bony window and no damage to the enamel organ (Figs. 2, 4A, B) in 8 out of 10 animals used to validate the surgical procedure prior to the present drug and tracer study.

Inaccurate anterior positioning resulted either in compression by bone debris (Figs. 4C, D) or focal destruction (Fig. 4E) of the enamel organ while, posteriorly, complete perforation of the thin alveolar bony walls occurred. The buccal (Fig. 2B) wall of the hemimandible is partially removed revealing the opposing lingual (L) aspect. Material infused into the hole diffuses along the periodontal space towards (arrows) the apical end of the incisor. Sections were cut along the longitudinal axis of the tooth (see Fig. 3). In the majority of cases, the hole (arrowhead) is positioned a short distance away from the enamel organ, resulting in no mechanical damage to the tooth (Figs. 4A-4B). Occasionally, bone debris (asterisk), created during drilling, compress part of the tooth surface causing minor focal tissue changes (Figs. 4C-D). Fig. 4D is an enlargement of the boxed area in (C). If the mandibular reference points are not respected, drilling can occur over the tooth organ inducing major tissue damage (Fig. 4E). The alterations, however, are generally localized to the area where the bur contacts the tooth. (inset) Occasionally, a layer of matrix resembling acellular afibrillar cementum (between small arrows) is deposited at the ameloblast surface near the site of damage (Am, ameloblasts; Ocl, osteoclast; Od, odontoblasts; P, pulp; PT, periodontal tissue).

Inflammatory cells infiltrated the damaged enamel organ and a cementum-like substance was deposited in the

forming extracellular matrix (Fig. 4E inset). Surprisingly. the enamel organ surrounding the site of tissue damage appeared to develop normally.

Morphological alterations induced by vinblastine sulfate

Continuous exposure of the dental organ to vinblastine sulfate for 3 days affected tooth eruption such that the treated incisor was about 1 mm shorter than the contralateral tooth (Fig. 5A). The incisor on the treated side (R) is shorter than the contralateral (L) tooth (Fig. 5A). The drug has diffused throughout the tooth affecting the enamel organ (EO), the pulp (P) and the root analog surface (RA) (Fig. 5B). (inset) Numerous mitotic Figs. (arrowheads) are present in the pulp. (Fig. 5C) The organization of the enamel organ (EO) as well as the production of enamel matrix (Fig. 5E) by ameloblasts (Am) are affected in the early part of amelogenesis (compare Figs. 5C-D). Ameloblasts that differentiated prior to administration of the drug show a normal organization, however, groups of odontoblasts (Od) appear to have degenerated (bracket) (Fig. 5D). (Fig. 5E) A focal alteration of dentin (Fig. 5D) production and mineralization (dashed lines) is sometimes observed on the root analogue surface (RA). No structural changes were observed on the contralateral tooth (PT, periodontal tissue) (Fig. 5F).

It is to be noted that none of the rats used in initial studies aimed at validating the surgical procedure (no minipumps placed) as well as those used for tracer studies showed a difference in length between the treated and contralateral, untreated incisor. Cell organization and function were also altered (Figs. 5B-E). The Golgi apparatus of secretory stage ameloblasts was fragmented, clusters of secretory granules were found throughout the cell body (Fig. 6A), and endosomal/lysosomal elements were abundant (Fig. 6B).

These cell compartments were all immunoreactive for amelogenin (Fig. 6). Local infusion of vinblastine sulphate through the bony window results in the typical cellular changes induced by systemic injection of this anti-microtubular agent (see Nanci et al. 1987). The Golgi apparatus (Golgi) is fragmented, secretory granules (sg) accumulate throughout the cell body, and numerous lysosome-like elements (asterisks) and multivesicular bodies (mvb) appear. Secretory stage ameloblasts also release material (arrowheads) ectopically along their basolateral surfaces. Immunoreactivity for amelogenin is present in all these compartments (N, nucleus) (Fig. 6).

Tomes' processes showed very few or no secretion granules (Fig. 7). These were abundant and intensely immunoreactive for amelogenin in contralateral ameloblasts (Fig. 7B). As expected, vinblastine results in the loss of secretory granules (sg) from Tomes' processes (Tomes) only on the treated side, demonstrating the localized action obtained by infusing drugs through the bony window. Secretory granules and the enamel matrix are immunoreactive for amelogenin.

The organization and shape of early secretory stage ameloblasts were disrupted, and there was ectopic release of enamel proteins along their baso-lateral surfaces (Fig 6A). Groups of odontoblasts showed signs of degeneration, even though the appearance of the overlying dentin seemed normal (Fig. 5D). There were abundant mitotic cells in the pulp and periodontal tissue (Fig. 5 inset). In one case, focal alteration of dentin production and mineralization on the root analogue surface were also observed (Fig. 5E). Tissues of the contralateral hemimandible appeared normal and showed none of the above cell and matrix alterations (Figs. 5F, 7B). The duodenum, kidney and parotid cells also revealed no structural changes.

Distribution of tracers

Dinitrophenol-tagged albumin was found in endosomal/lysosomal elements of periodontal fibroblasts and in the interstitial fluid surrounding them (Figs. 8), and less frequently in pulp cells and odontoblasts (Fig. 9, N, nucleus). The tracer is taken up by fibroblasts into their lysosomal/endosomal elements (arrowheads). More tracer molecules are immunodetected extracellularly (Fig. 8A) near the bone window than (Fig. 8B) at a distance (~3mm) from the hole (Coll, collagen fibrils; N, nucleus).

There was also abundant immunoreactivity around osteoblasts and in osteoid near the site of infusion (Fig. 10), but the density of labeling associated with bone diminished significantly away from the bony window. Since these cells are separated from the pulp by mineralized layers of dentin and enamel, the endocytosed molecules must have diffused through the multiple cell layers of the enamel organ in order to reach the ameloblasts (A, nonosmicated; B, osmicated; N, nucleus).

DNP-albumin was also detected in multivesicular bodies of early secretory stage ameloblasts (Fig. 11). Some tracer is also immunodetected extracellularly (arrows) among the pulp cells (N, nucleus).

Although some labeling was sporadically found in liver cells, only few, randomly distributed gold particles were observed on tissue sections of the contralateral tooth incubated with anti-DNP antibody. Control incubations with protein A-gold alone resulted in few gold particles, randomly distributed throughout the tissue section.

Fetuin-gold was predominantly found at the site of drilling but some complex was detected in the periodontal tissue along the incisor up to 5 mm away from the bony window. Gold particles accumulated within clotted matrix

(Fig. 12A) and in endosomal/lysosomal elements of macrophages and neutrophils in the bony defect. Periodontal fibroblasts in proximity of the window (Figs. 12, 13A) and, less frequently and intensely, pulp cells (Fig. 13B) also showed the intracellular presence of tracer. No gold particles were detected in the enamel organ, in the contralateral tooth or other distant tissues sampled.

Distribution of tracers

Gene transfer has been successfully performed in a large variety of cells such as myotubes, hepatocytes, endothelial cells, airway epithelial cells and a variety of neuronal cells (H. Okada et al., 1996, Gene Therapy, 3:957-964). A number of gene therapy vectors have been developed and described and their efficiency proven both *in vitro* and *in vivo* (V. Budker et al., 1996, Gene Therapy, 3:593-598). These include the viral vectors such as the adenoviruses, retroviruses and herpes viruses, and the non-viral vectors which include the use of cationic lipids as well as naked DNA (AK Lalman et al., 1996, Gene Therapy, 3:588-592).

In order to efficiently transduce cells of the odontogenic organ and associated periodontal tissues of the rat incisor, we are currently applying several of these gene therapy vectors through the surgical window. In preliminary experiments, we have recombinant adenovirus vector which encodes for the marker gene beta-galactosidase. This vector has the capacity to infect a large array of cell types, it is more stable than its counterpart the retrovirus and it is possible to obtain high viral concentrations (10^{12}/Ml).

In our initial experiments, we have used concentrations ranging from 10^{8-9} in order to transduce cells of the odontogenic organ. A 2001D osmotic minipump and vinyl outlet tubing were filled with recombinant

adenoviral vector diluted in sterile PBS. The pump was immediately placed into the animal and connected to the surgical window as describes above. Three days later, the animals were sacrificed, and the incisor was conventionally-processed for beta-gal staining. Other tissues such as the liver, duodenum and parotid gland were also collected and stained in order to assess systemic diffusion of the virus. Although enamel showed some nonspecific staining, there was a more pronounced staining at the apex of the odontogenic organ which decreased incisally away from the site of infusion (Fig. 14). Only background staining was detected in the contralateral untreated tooth and no reactivity was seen in the other organs. These results demonstrate the effectiveness and specificity of our approach in targeting the enamel organ. We are currently testing retroviralmediated gene delivery as well as cationic lipid gene delivery systems in order to assess their efficiency to transduce the tooth organ and associated tissues system.

DISCUSSION

In order to address molecular mechanisms involved in normal and pathological calcified tissue formation, we have developed an *in vivo* approach to experimentally access and manipulate the odontogenic organ of a rodent incisor and its associated periodontal tissues. This tooth was chosen since it offers the possibility to investigate developmental processes and the deposition of both collagenous and noncollagenous mineralized matrices in a well-defined temporo-spatial sequence. An osmotic minipump, connected to a bony window in the alveolar bone overlying the apical end of the tooth, allowed controlled and continuous administration of experimental agents to the tooth organ and its surrounding tissues.

In order to avoid complications associated with previous experimental approaches for accessing the tooth organ, we have positioned our surgical access on the buccal side of the alveolar bone, overlying the periodontal tissue just posterior to the apical end of the mandibular incisor. This site allows access to both the tooth organ and a zone of active bone remodeling along the posterior wall of the hemimandible. From this site, the experimental agents can diffuse to the enamel organ and its adjacent structures via the periodontal space separating the alveolar bone and the tooth. Damage to the odontogenic organ was observed in very few cases of operated animals and was, in general, due to anatomical variability. Most of the damages were confined to the apical end of the tooth and did not seem to significantly alter tooth formation and/or eruption. Damage to the enamel organ in some cases resulted in the production of a matrix resembling that found at the enamel-free area on the cusp tips of rodent molars, an observation relevant to the proposed epithelio-mesenchymal transformation of enamel organ cells during odontogenesis (Bosshardt and Nanci 1998). Damage to the enamel organ can also be avoided by positioning the hole at the base of the molars along the root analogue surface of the incisor. Beside direct targetting of selected regions of the incisor, this allows to access cellular and matrix events around the molars.

Use of minipumps is preferred to administer experimental agents. In contrast to systemic injection or local microinjection, it is possible to deliver with minipumps relatively large volumes of an experimental agent over a precise period of time. Vinblastine sulfate was infused over a 3 day period to obtain local tissue alterations without any systemic effects. The changes obtained in the enamel organ are consistent with

previously published data using local or systemic injection of vinblastine (Nanci et al. 1987). In addition, alterations induced in the pulp, dentin, cementum and periodontal tissue indicate that the drug diffuses locally and demonstrate the efficacy of our approach in targeting all the tissues of the tooth organ. Most importantly, the contralateral, untreated tooth and distant tissues such as duodenum showed no sign of being affected by the drug, thereby confirming that restricted and localized effects can be obtained. Indeed, the treated incisor was ~ 1 mm shorter than the contralateral tooth, an amount within the range but short of the expected eruption distance (1.9 mm) in mandibular incisors of rats weighing about 100g during a 3 day interval (calculated from Smith and Warshawsky 1975b). This difference suggests that the treatment reduced but did not stop tooth eruption. Although some diffusion of infused products into the circulatory system is likely to occur, the fact that very low amounts are required to obtain a local effect reduces greatly the potential and intensity of any systemic side effects. Indeed, even in the case of DNP-albumin, a circulating serum protein, there was no significant systemic redistribution.

It is generally accepted that the presence of protein outside the cell stimulates the ingestion of solutes from the interstitial fluid. The presence of tracers such as DNP-albumin and fetuin-gold in endosomal/lysosomal elements of ameloblasts, odontoblasts, osteoblasts and fibroblasts is consistent with the notion that most cells exhibit pinocytotic activity. Since secretory stage ameloblasts are separated from the pulp by mineralized layers of dentin and enamel, the presence of DNP-albumin found in these cells demonstrates that relatively large proteins can diffuse into the multiple cell layers of the enamel organ, as

previously shown following systemic injection (Nanci et al. 1996a). Accessibility of infused products to the various cells of the tooth and associated periodontal tissues is of utmost importance for gene activation/inactivation studies ("local" knock-out or transgenic) using molecular probes. Fetuin-gold complex is a larger tracer molecule which was found mostly in the extracellular matrix and was intensely phagocyted by inflammatory and fibroblastic cells in the immediate vicinity of the bony window. However, it did not diffuse across the enamel organ to be picked up by ameloblasts. Nonetheless, some fetuin-gold was detected in fibroblasts of the periodontal tissue as far away as 5 mm from the window. These tracer results indicate that the extent of tracer dissemination may vary depending on the size, the nature, interaction with matrix proteins, and its ability to elicit an inflammatory response.

In conclusion, the data presented herein validate our experimental approach and show that the various cells of the tooth as well as those of the surrounding tissues can be targeted. It may be advantageously applied to manipulate *in vivo*, using various therapeutic agents, the complex cellular and extracellular matrix events involved in the formation of both collagenous and noncollagenous calcified tissues. The ability to interfere with the genes responsible for the production of target proteins of the odontogenic organ and associated tissues may help advance our understanding of mineralized tissue formation and pathological alterations. Application of molecular probes through the bony window offers the possibility to activate/inactivate locally and selectively gene products for studies of function and treatment of disease. Such an approach is potentially less time consuming and costly than the genetic engineering of knock-out or transgenic animals, and may be particularly valuable in the case

where genetic alterations result in a lethal phenotype. Some molecular probes have already been used *in vitro* (antisense, Slavkin 1995) or by gross local injection (ribozymes, Lyngstadaas et al. 1995), have resulted in limited success, but have not yet been tested *in vivo*, in part due to their toxicity or generalized effects when injected systemically.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.